New Technologies for Bioartificial Organs

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ABSTRACT

Each year several hundred thousand Americans die because of organ failure. Organ transplantations have achieved remarkable success, but the source of human organs is limited. Only a small portion of patients can benefit annually from this method of treatment. Immunoisolation of living cells as bioartificial organs can be transplanted into human without the need for immunosuppression and its accompanying side effects; it allows cells from nonhuman species to be used thereby overcoming a limited supply of human cells available for encapsulation; and, the capsule can serve as an ideal cage to keep animal viruses from contacting the human host.

To achieve this promise, we have incorporated the pore size distribution into a new design of capsule for immunoisolation of living cells. In this model, the capsule wall is thicker and the pores are bigger than in the current systems. The larger pores will allow the immune system to enter the membrane, and the smaller pores inside the membrane will act as the traps to prevent or delay most of the immune system from passing all the way through to the inner volume of the capsule where the living cells resides. Limited animal studies have supported the advantages of this new entrapment model over the current model. Systematic studies to determine optimal capsule design for human transplantation are now feasible.

Key words: **Bioartificial Organs, Immunoisolation, Transplantation, Encapsulation, Living Cells**

INTRODUCTION

Immunoisolation of living cells for the use as bioartificial organs in humans have shown great potential.[1-3] It offers the following exciting possibilities: (i) cell transplantation without the need for immunosuppression and its accompanying side effects, (ii) transplantation of cells from nonhuman species (xenograft) to overcome the limited supply of donor cells, and (iii) an ideal cage to keep animal viruses from infecting the human host (if it is needed.) To achieve this promise, a capsule must be developed to protect both the cells and the host, and allow the cells to have normal physiological functions.

The capsule membrane must satisfy a set of stringent and dichotomous requirements. The membrane, to serve as an immunoisolation device, must be able to keep the host immune system away from the living cells and keep the donor virus from the host, yet allow the nutrients, oxygen and protein to pass through without much impedance. The membrane must be biocompatible to the host and to the cells it encloses. It also needs to be strong enough to survive the handling, transplantation, and the hostile environment inside the human body.

MODEL

The effectiveness of immunoisolation is closely tied to the capsule's permeability, or more precisely, the pore size of the capsule membrane. It has been suggested, and argued forcefully, that with proper selection of cutoff pore sizes, the polymer membrane can serve as a barrier, keeping the immune system out, donor virus in, and allowing the oxygen and hormone to pass through without much resistance. (Fig. 1)

Most of the current capsule designs are based on this principle[4-8], however, there is a fundamental flaw in this physical picture. The barrier model ignores the fact that capsule pore sizes are inhomogeneous and have very long tails due to the nature

of polymer membranes formation. For instance, based on our dextran permeability measurements, the apparent pore size distribution of the capsular membrane has a cut off at 117Å (230 KDa)[9-11] as shown in Figure 2. The shaded area of Figure 2 represents the pore size tail. However, if we were to replot Figure 2 as the pore surface area distribution rather the pore size distribution, (Fig. 3), the tail is no longer a tail anymore. This large tail surface area may have contributed to the inconsistent conclusions in the literature.[12-20] For the capsule to provide immunoprotection for the living cells, the bulk of the surface area must have a pore size smaller than the antibody complement component.[13] This suggests that unless a barrier capsule has a step function cutoff at 300(Å) in pore size diameter and defect free, the effectiveness of the capsule as an immunoisolation device will be compromised, and less than optimal capsules will be used in human clinical trials.

In this paper, we propose to incorporate the pore size distribution into a new design of capsule for immunoisolation of living cells. In this model, the capsule wall is thicker and the pores are bigger than in the barrier model. The larger pores will allow the immune system to enter the membrane, and the smaller pores inside the membrane will act as the traps, to prevent or delay most of the immune system from passing all the way through to the inner volume of the capsule where the living cells resides. Thus, we have designed a capsule that allows nutrients to enter the capsule, but prevents immune systems from attacking the cell.

An addition advantage of this model is that the parameters of the capsule can optimized independently[9] to allow the cells to have normal physiological functions. The capsule membrane serves as an immune system **entrapper** than as a barrier.

Figure 4 is the schematic drawing of our entrapment model (notice that the wall is considerably thicker than Fig. (1)). The immune system, in a diluted state, passes though the capsule as a result of "random walk motion". The random walk statistics is the bedrock of Statistical Mechanics and Thermal Physics.[21] In this paper, we will apply the same methodology to this immunoisolation problem. For the sake of simplicity, we shall study the motion in one dimension, and assume the probability to

go forward and backward of equal steps are the same. After a total of N such steps, the immune system can be found at

$$X = m\Delta \tag{1}$$

Where $m = n_f - n_b$, and $n_f + n_b = N$. n_f represents the step forward, n_b the step backward. Δ is the length of each step and can be approximated to be the average pore size of the membrane.

The probability of finding the immune system at a given position $m\Delta$ away from the origin is

$$P_N(m) = \frac{N!}{\left(\frac{N+m}{2}\right)!\left(\frac{N-m}{2}\right)!}\left(\frac{1}{2}\right)^N \tag{2}$$

This is a Guassian distribution curve centered at zero, as one would expect, due to the symmetry. If the probability to go forward and backward are not the same, the center will be off zero, but general characteristics of the curve will be the same.

With some math, the width of the range which the immune system has travelled after N steps is found to be

$$\overline{(\delta x)^2} = \overline{(\delta m)^2}\,\Delta^2 = N\Delta^2 \tag{3}$$

Where $\delta x = x - \bar{x}$ and $\delta m = m - \bar{m}$.

If we assume the immune system is originally located at the surface of the capsule, and the distance needed to travel is the membrane thickness T. The eq. (3) can be rewritten as

$$\frac{T^2}{\Delta^2} = N \qquad (4)$$

We further simplify the problem by assuming the immune system passes through the large pores without any resistance, and is retained by the small pores for a time period of "τ".

We can now write the total time "Γ " required for the immune system to pass through the membrane with a thickness "T" as

$$\Gamma \sim \frac{T^2}{\Delta^2} f \tau \qquad (5)$$

The "*f* " is the ratio between surface areas of small pores and large pores. Using Equipartition theorem, the random walk motion in three dimensions can be written as

$$\Gamma \simeq 3\frac{T^2}{\Delta^2} f \tau \qquad (6)$$

Using antibody complement component as a model, the estimated entrapment time for the SA-PLL-SA barrier capsule (110 KDa, 2 μm wall thickness) is in the order of days. This might help to explain why most transplantation has failed for pre-sensitized animal models like NOD mice. More importantly, this might suggest the current barrier model will have limited success in treating Type I diabetic disease in humans.

EXPERIMENTS

Immunoisolation of living cells for the use as bioartificial organs in human has wide applications. Currently, there are active studies on artificial pancreas, artificial liver, artificial parathyroid, and others.[22] In this paper we will test our model as artificial pancreas. We have developed a new capsule after studying more than a thousand polymer combinations, we have settled on sodium alginate (SA), cellulose sulfate (CS), Poly(methylene-co-quinidine) (PMCG), calcium chloride ($CaCl_2$), and sodium chloride (NaCl).[9-10]

Capsule Studies

i) Biocompatibility

We have studied the fibrotic growth on our new capsules as a function of polycation concentration with C57 and NOD mice. We found the biocompatibility of the capsule inside normal C57 mice was not very sensitive to minor polycation concentration changes (Fig. 5A), however, the NOD mice model had a different response all together. We found that a small change in the concentration of polycation can alter the capsules inside the NOD mice from clumped to free floating to breakage. (Fig. 5B) The effect of autoimmune disease on the biocompatibility of the capsule is self-evident here.

ii) Nutrient, Oxygen, and Hormone Transport

In our animal studies with the chemically induced diabetic mice (STZ), we have noticed the eventual failure of the encapsulated islets more likely resulted from either nutrient or oxygen deficiency. In order to study the effect of pore size on nutrient transport, we have transplanted different sets of encapsulated islets (230 KD, 80 μm

and 350 KD, 80 μm; 350 KD 40 μm and 80 μm, respectively) into the same animals. The islets were retrieved at given intervals, separated and perifused. The insulin response of the islets encapsulated in different pore size capsules were compared. (Fig. 6) Clearly shown, are the advantages of encapsulating islets in capsules with larger pore sizes, and with a thinner membrane.

iii) Mechanical strength

The mechanical strength of the membrane must be strong enough to survive the handling, transplantation, and the hostile environment inside the human body. The mechanical strength of capsules was measured by placing an increasing uniaxial load on the capsule until the capsule burst. The capsule mechanical strength, a function of membrane thicknesses, can be adjusted anywhere from a fraction of a gram to tens of gram load to meet the needs of transplantation without altering the permeability of the capsule.[9] The rupture load versus the product of membrane thickness and capsule size and demonstrates that the entrapment capsule has much greater flexibility in mechanical strength than barrier capsules system.[23]

iv) Capsule Permeability

Measurement of capsule permeability was improved by utilizing two complementary methods: i) size exclusion chromatography (SEC) with dextran molecular weight standards, and ii) a newly-developed method to assess permeability of a series of biologically-relevant proteins using encapsulated protein A-sepharose (PAS). By combining these methodologies to measure permeability and component concentration manipulations to control capsule permeability, a series of capsules with a range of permeabilities (40 kDa - 230 kDa, based on dextran exclusion measurement) was developed and characterized.

The apparent pore size of the capsular membrane was determined by size exclusion chromatography (SEC) that measures the exclusion of dextran solutes from

the column packed with microcapsules.[11] Using neutral polysaccharide molecular weight standards, it is possible to evaluate the membrane properties under the conditions when solute diffusion is controlled only by its molecular dimension. Based on the measured values of solute size exclusion coefficients (K_{SEC}) and known size of solute molecules, one can estimate the membrane pore size distribution (PSD) as shown in Figure 2. The capsular membrane excluded dextran molecules with molecular weight above 230 kDa which corresponds to $R_\eta \geq 117$ Å.

v) Capsule Diameter Effect

Different diameters capsules (0.8 mm and 0.5 mm, respectively) were transplanted into the same animals to study their effects on nutrient transport. The islets were retrieved at given intervals, separated and perifused. The insulin responses of cells encapsulated in different diameters have been found to be the same within the error bar. (Data not shown) This suggests the capsule size may have minor effect on the transport mechanism. This can be understood by viewing the capsule as part of a total impedance system. The nutrient concentration gradient immediately adjacent to the islets is strongly affected by the presence of a membrane, and less by the position of the membrane.

Animal Trials

Published data[9,13,24-25] indicates encapsulated rat islets, regardless of capsule model, readily reversed chemically induced (Streptozotocin) diabetics in mice and islets function maintained for many months. On the other hand, with few exceptions[14,15] long term normoglycemia has not be demonstrated in NOD mice by islets encapsulated in the barrier model.[13,16-20] Therefore, the effectiveness of our entrapment model can best be demonstrated in NOD mice. Figure 7 shows the reversal of diabetic NOD female mice up to 241 days. This is in contrast to the few weeks reversal for NOD mice with barrier model. Furthermore, we have noticed the eventual failure of encapsulated rat islets transplanted into NOD mice are likely to

have resulted from an immune or autoimmune attack. Capsules retrieved from those animals were clumped and demonstrated marked fibrosis around the capsule surface. This indicates the eventual breakthrough of the immune system in the time frame predicted by eq. (6). This trial data supports the physical picture of immunoprotection of our new entrapment model.

DISCUSSION

Bioartificial organs for humans, based on Immunoisolation of Living Cells, have great potential in healthcare. Capsules can keep the host immune system from entering and the animal virus from exiting it, and yet allow entrance and exit of substances required for cell survival and function. The concept of such a barrier is rather straightforward, and many immunoisolation capsule designs are based on this principle, however, there is a fundamental flaw in this model. The pore size distribution of capsular membrane is inhomogeneous, and has very long tail. This heterogenicity in pore size may have limited the success of immunoisolation capsules in human transplantation.

In this paper, we have proposed a new physical picture on the mechanism of immunoisolation to explain. We have developed a new entrapment capsule based on the new Physical Picture.

In the entrapment capsule, with the right choice of pore size and membrane thickness, the nutrient, hormone oxygen are free to enter the membrane but immune system are entrapped and delayed from attacking the cells. Limited animal studies have supported the advantages of the entrapment model over the barrier model. Systematic studies to determine optimal capsule design for human transplantation are now feasible.

EXPERIMENTAL PROTOCOL

Assessment of Capsule Permeability

To assess capsule permeability to immunologically-relevant proteins like IgG, protein A-sepharose (PAS, Sigma Chemicals, St. Louis, MO) was encapsulated. The empty capsules and capsules containing ~18 µl of packed PAS were equilibrated in phosphate-buffered saline (PBS) with 0.2% Tween 20 and 0.2% bovine serum albumin. Iodinated IgG (approximately 10,000 cpm; New England Nuclear, Wilmington, DE) was added and aliquots removed at selected time points over 24 hours. After removal at each time point, the encapsulated PAS was washed five times with 4 ml of PBS. The amount of IgG that had entered the capsule was quantified in a gamma counter. Unencapsulated or free PAS served as a positive control. This method of permeability assessment can be adapted for measuring entry of almost any protein since the only requirements are an antibody against the protein of interest (in this case the PAS would be preincubated with the antibody before encapsulation) and the ability to radiolabel the protein.

Assessment of Insulin Secretion

Insulin secretion by encapsulated rat islets was evaluated in a cell perifusion apparatus with a flow rate of 1 ml/minute with RPMI 1640 with 0.1% BSA as a perfusate. Encapsulated islets were perifused with 2 mM glucose for 30 minutes and the column flowthrough discarded. Three minute samples of perfusate were collected during a 30 minute perifusion of 2 mM glucose, a 30 minute perifusion of 20 mM glucose + 0.045 mM IBMX, and a 60 minute perifusion of 2 mM glucose. Samples were assayed in duplicate for insulin using Coat-a-Count kits (Diagnostic Products Corporation, Los Angeles, CA) with a rat insulin standard. The amount of insulin secreted was normalized for the number of islets.

Animal Studies

Pancreatic islets were isolated from male Sprague-Dawley rats (250-275 g, Harlan, Indianapolis, IN). Briefly, the pancreatic duct was inflated with a solution of Hank's Balanced Saline Solution (HBSS) containing collagenase (Boehringer-Mannheim, Collagenase P, Indianapolis, IN). Groups of 3 pancreases were digested in 2 mg collagenase/ pancreas in HBSS for 6-13 minutes at 37^{o}C using a wrist-action shaker. The digestion was stopped by the addition of cold HBSS with 10% Newborn Calf Serum and shaken vigorously for 10-15 seconds. The digested material was washed three times with cold HBSS and filtered through a wire mesh cell strainer to remove undigested material. Pancreatic islets were separated using a Histopaque-1077 gradient and stored in University of Wisconsin storage solution for 19-24 hours before encapsulation. Encapsulated rat islets were transplanted into the peritoneal cavity of diabetic mice (either strepzotocin-induced or NOD mice). Diabetes was induced in normal C57/Bl6 mice by the intraperitoneal injection of strepzotocin (200 mg/kg) 3-7 days prior to transplantation of encapsulated islets. NOD mice were purchased from Taconic Laboratories (Germantown, NY) and bred in microisolator cages with autoclaved bedding and water. Between 18-24 weeks of age, 50-75% of females from this colony became diabetic. When diabetes was present for 2-4 weeks, female NOD mice received a transplantation of encapsulated islets. Approximately 1000 encapsulated rat islets (0.8 mm capsule with 0.1 mm wall thickness and an exclusion limit of 230 KDa), in a packed capsule volume of 0.2-0.5 ml, were sterilely transplanted intraperitoneally into metofane-anesthetized mice. The blood sugar of mice was measured using a one-touch glucometer using blood obtained from the retroorbital plexus or a tail vein.

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FIGURE CAPTIONS

Fig. 1 **Barrier model.**

The immune system is excluded from entering the capsule, yet allowing the nutrients, oxygen and protein to pass through without much impedance.

Fig. 2 **Pore size distribution of capsule membrane.**

Apparent pore size distribution (PSD) of SA/CS/PMCG microcapsules as estimated based on dextran viscosity radius R_η are shown. The SA/CS/PMCG capsules were prepared from the mixture of 0.6% SA and 0.6% CS; the polycation solution contained 1.8% PMCG, 1% $CaCl_2$ and 0.9% NaCl (1 min reaction time). The exclusion limit of this capsule, as determined by SEC with dextran was 230 KDa. The dashed area represents the pores larger than the cutoff limit. The dotted line is the radius of IgG.

Fig. 3 **Surface area distribution of capsule membrane.**

The surface area distribution curve of capsule membrane is derived from multiplying the surface area of each pore with the apparent pore size distribution curve above. The dashed line is the cutoff pore size, and the dotted line is the radius of antibody complement component. The solid line is to guide the eye, and represents no theory.

Fig. 4 **Entrapment model.**

In the entrapment model, the capsule wall is thicker and the pores are bigger than in the barrier model. This allows the immune system to freely

enter the membrane. However, there are sufficient small pores inside the membrane which will act as the traps, to prevent or delay most of the immune system from passing all the way through to the inner volume of the capsule where the islet resides.

Fig. 5 **Biocompatibility of empty capsules in C57 mice and NOD mice.** These capsules were prepared under identical processing steps, but different PMCG concentrations.

Panel A. [TOP] Capsules retrieved from Normal C57 mice with PMCG concentrations varying from 1.0, 1.6, 1.8, 2.0.
Panel B. [BOTTOM] Capsules retrieved from Normal NOD mice with PMCG concentrations varying from 1.6, 1.8. (The 1.0 and 2.0 capsules could not be retrieved from the animals.)

Fig. 6 **Insulin response of the islets.**
Islets encapsulated in two different pore size capsules (230 KDa and 350 KDa) were transplanted into the same C57 mice. The islets were retrieved, separated, and perifused at 2 week and 4 week intervals.

Fig. 7 **Transplantation of encapsulated rat islets reverse diabetes.**
Encapsulated rat islets were transplanted into the peritoneal cavity of female NOD mice which had developed spontaneous diabetes. Four diabetic mice with 1.8 PMCG capsules transplantation survived up to 60 days, one with 1.6 PMCG capsules was normoglycemic 241 days following transplantation.

IMMUNOISOLATION OF LIVING CELLS

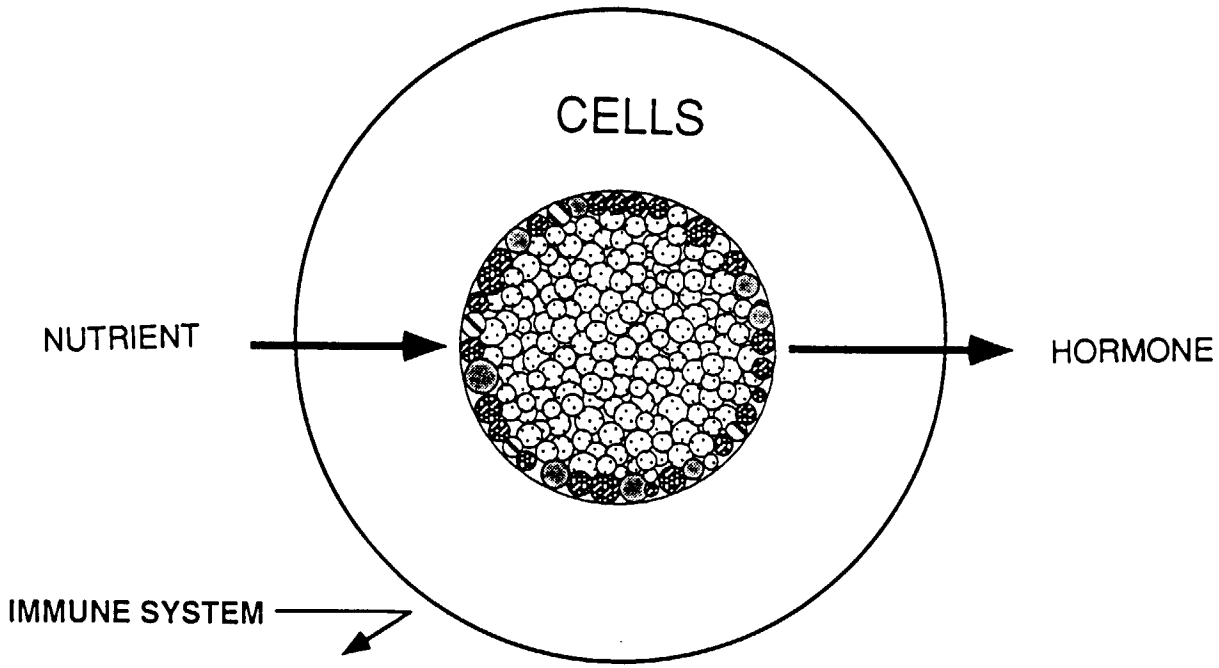

Fig. 1 Barrier model.

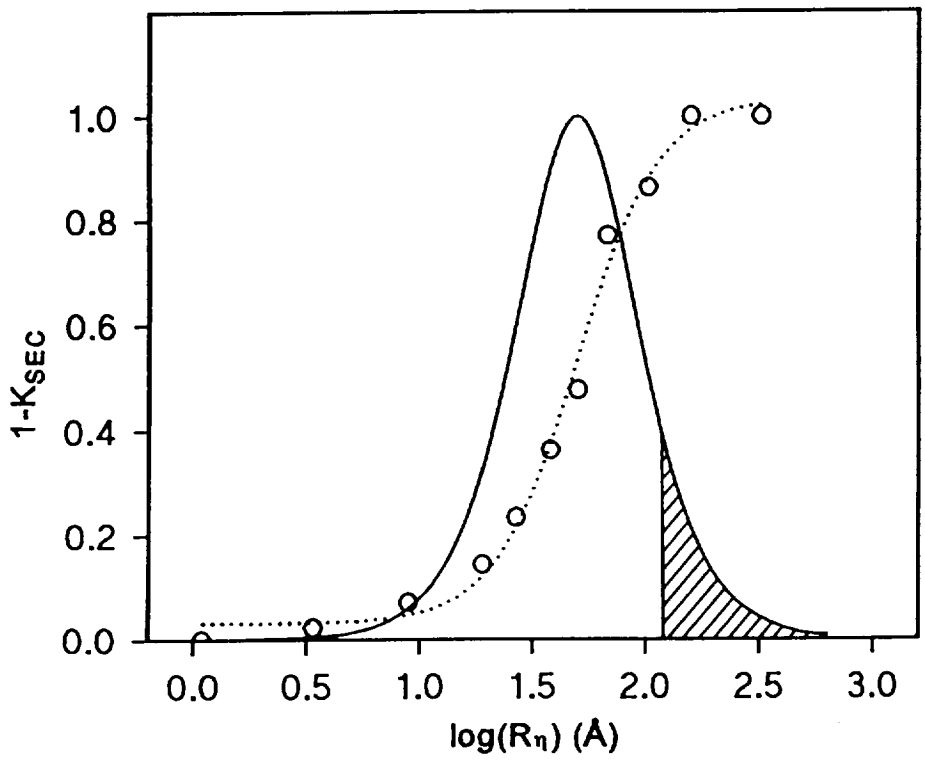

Fig. 2 Pore size distribution of capsule membrane.

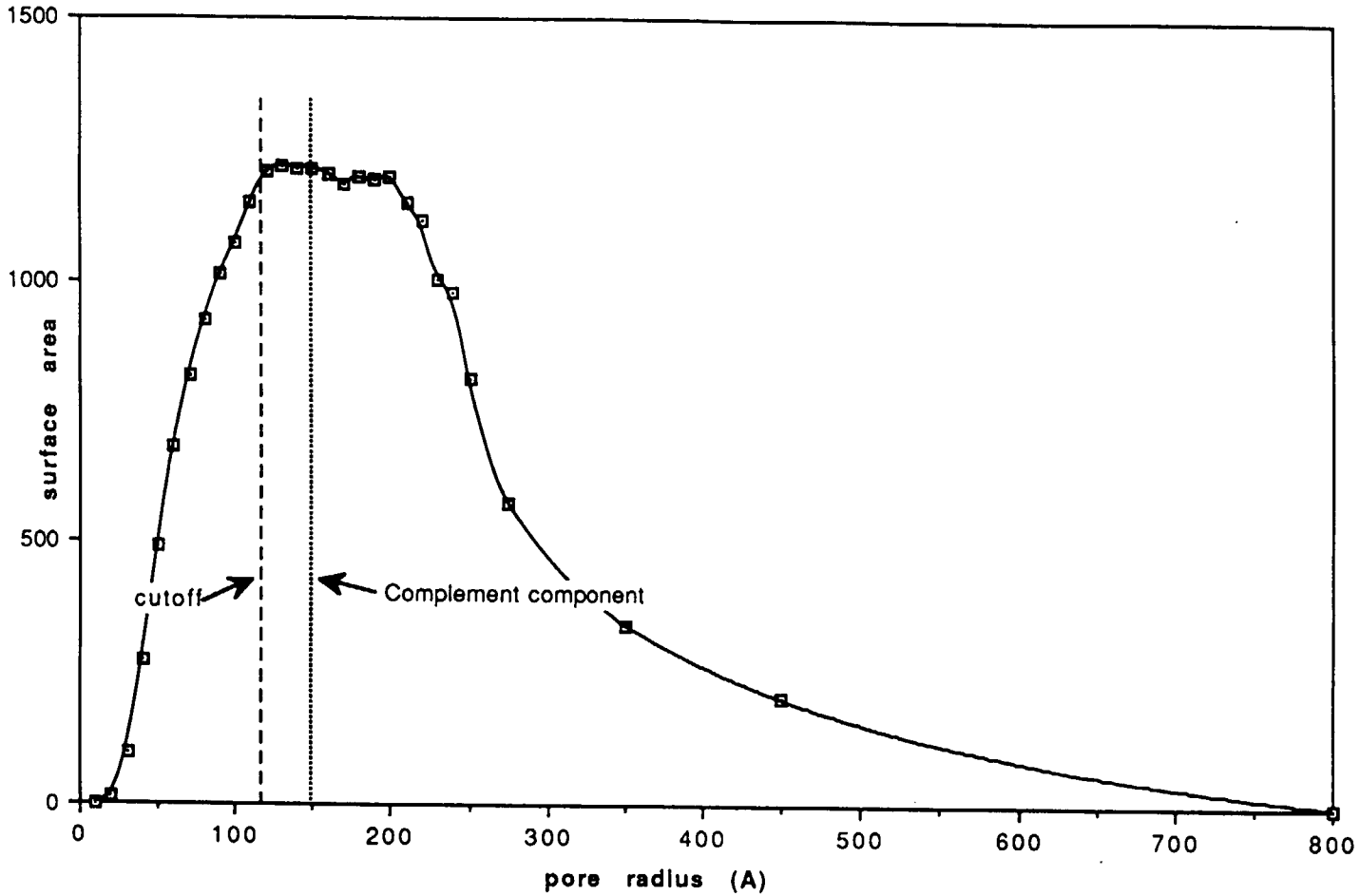

Fig. 3 Surface area distribution of capsule membrane.

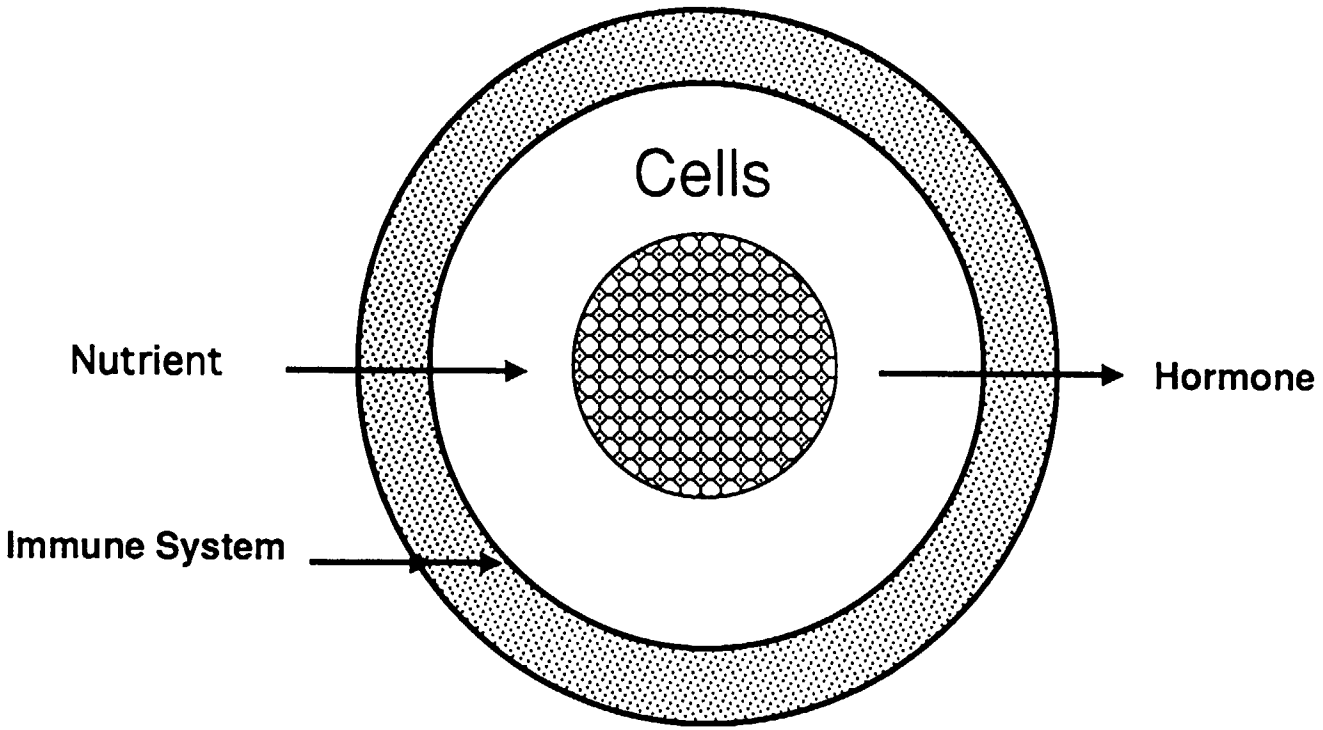

Fig. 4 Entrapment model.

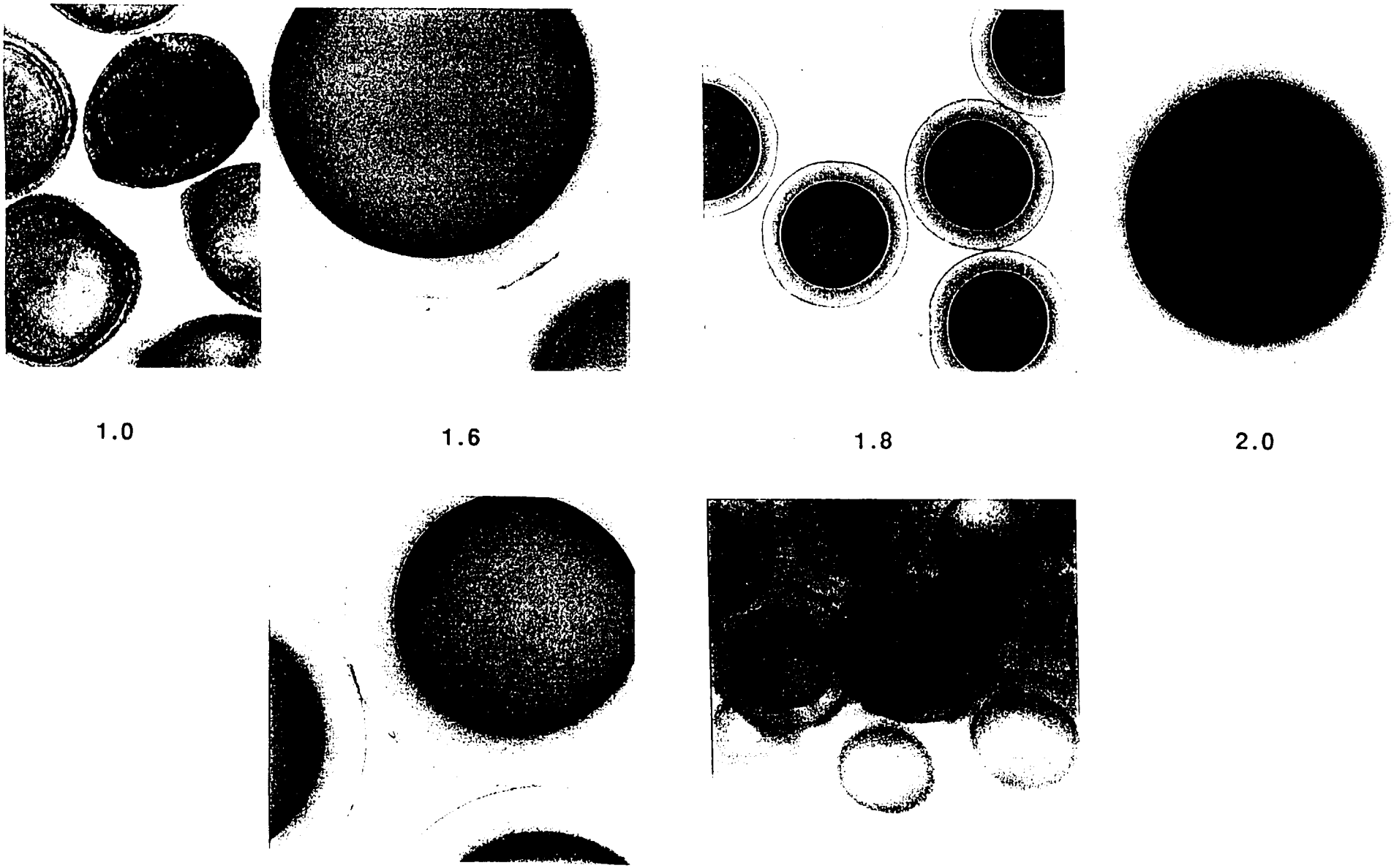

Fig. 5 Biocompatibility of empty capsules in C57 and NOD mice.

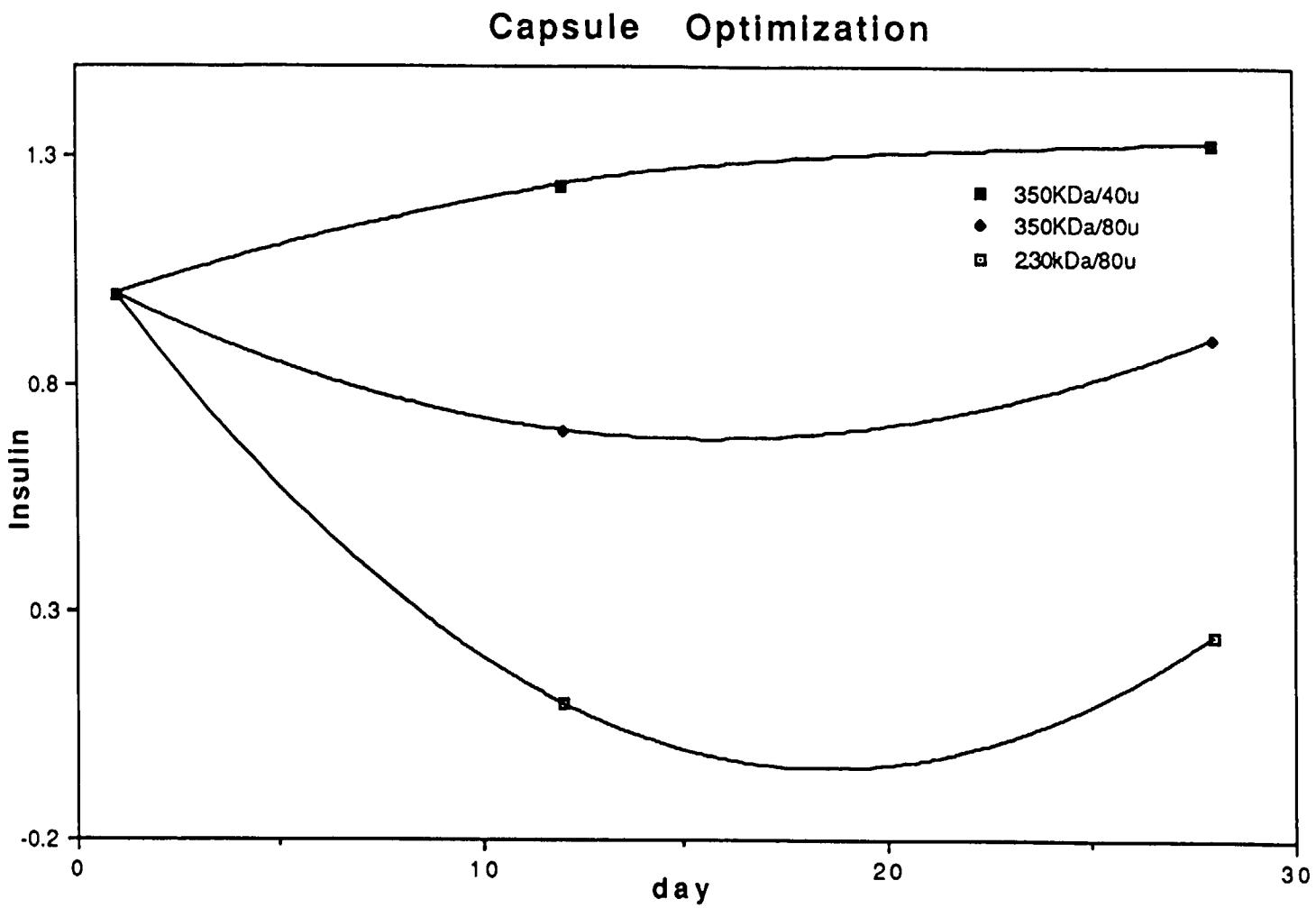

Fig. 6 Insulin response of the islets.

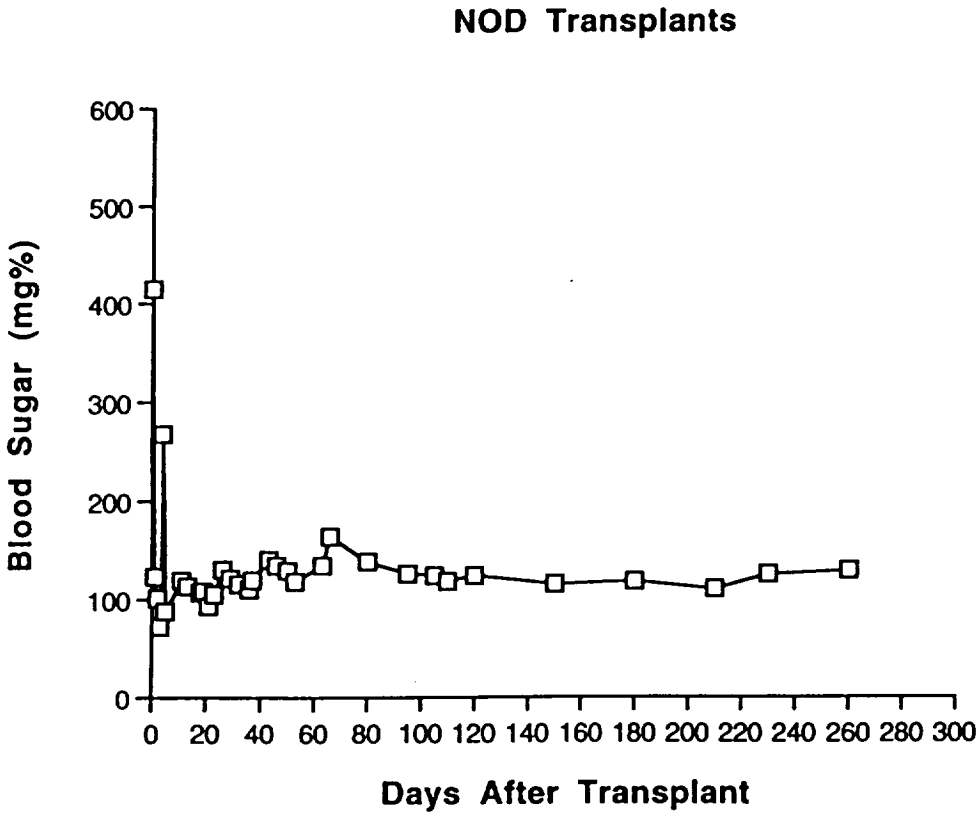

Fig. 7 Transplantation of encapsulated rat islets Reverse diabetes.